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Plant dihydroorotase

The present invention relates to the identification of plant dihydroorotase as a novel target for herbicidal active ingredients. The present invention furthermore relates to DNA sequences encoding a polypeptide with dihydroorotase (EC 3.5.2.3) activity. Also, the invention relates to the use of a nucleic acid encoding a protein with dihydroorotase activity of vegetable origin for the generation of a test system for identifying herbicidally active dihydroorotase inhibitors, and to inhibitors of plant dihydroorotase identified using these methods or this assay system. In addition, the present invention relates to a DNA sequence encoding a polypeptide with dihydroorotate dehydrogenase activity and to its use as auxiliary enzyme in a molecular assay system. Furthermore, the invention relates to the use of the nucleic acid encoding plant dihydroorotase for the generation of plants with an increased resistance to dihydroorotase inhibitors. In addition, the invention relates to a method of eliminating undesired vegetation, which comprises treating the plants to be eliminated with a compound which specifically binds to, and inhibits the function of, dihydroorotase encoded by a DNA sequence SEQ-ID No. 1 or by a DNA sequence hybridizing with this DNA sequence.

Plants are capable of synthesizing their cell components from carbon dioxide, water and inorganic salts.

This process is only possible by exploiting biochemical reactions for the synthesis of organic substances. Nucleotides, being constituents of the nucleic acids, must be synthesized de novo by the plants.

Not only the enzyme reactions of the *de novo* purine biosynthesis, but also the enzyme reactions of the *de novo* pyrimidine biosynthesis, are important for regulating the nucleotide metabolism. One of these enzymes is dihydroorotase. The enzyme catalyzes the elimination of water from carbamoyl aspartate and the cyclization to give dihydroorotate. The subsequent enzyme dihydroorotate dehydrogenase converts dihydroorotate into orotate via a redox reaction, see Figure 1.

Genes which encode dihydroorotases were isolated from a variety of organisms. Complete cDNA sequences are known from bacteria (GenBank Acc. No. M97254, Pseudomonas putida, X84262 Lactobacillus leichmannii, AE000207 Escherichia coli, M97253 Pseudomonas putida, P74438 Synechocystis). In eukaryotes,

dihydroorotase is a component of a multifunctional enzyme complex which is localized on an coding sequence (for example X03881 Drosophila melanogaster). In yeast, too, dihydroorotase is present in a multi-enzyme complex (Souciet et al., Mol. Gen. Genet. 207 (2-3), 314-319 (1987)). In plants, dihydroorotase is not a component of a polyfunctional polypeptide, but, similarly to what is the case in E. coli, exists as a separate enzyme. A plant dihydroorotase has hitherto only been isolated from Arabidopsis thaliana (Genbank Acc. No. AF000146; Zhou et al., Plant Physiol. 114 (1997), 1569.).

The demonstration that an enzyme is suitable as herbicide target can be shown, for example, by reducing the enzyme activity by means of the antisense technology in transgenic plants. If this results in reduced growth, it can be concluded that the enzyme, whose activity is reduced, is suitable as site of action for herbicidal active ingredients. This was shown by way of example for acetolactate synthase in transgenic potato plants (Höfgen et al., Plant Physiology 107 (1995), 469-477).

It is an object of the present invention to prove that dihydroorotase in plants is a suitable herbicidal [sic] target, to isolate a complete plant cDNA encoding the enzyme dihydroorotase and its functional expression in bacterial or eukaryotic cells, and to generate an efficient and simple test system for carrying out inhibitor-enzyme binding studies.

We have found that this object is achieved by isolating a gene encoding the plant enzyme dihydroorotase, generating dihydroorotase antisense constructs, and functionally expressing dihydroorotase in bacterial or eukaryotic cells.

The present invention firstly relates to a DNA sequence SEQ-ID NO:1 comprising the coding region of a plant dihydroorotase from Solanum tuberosum (potato), see Examples 1 and 2.

The present invention furthermore relates to DNA sequences which are derived from this SEQ-ID NO:1 or hybridize herewith and which encode a protein which has the biological activity of a dihydroorotase.

Plants of the ROSa lines, which carry a dihydroorotase antisense construct, have been characterized in greater detail. The plants exhibit different degrees of growth retardation. The plant line ROSa-40 is affected to such an extent that no tubers are formed. Plants of this line are not viable in the greenhouse and must be maintained in vitro. A correlation between growth retardation and

reduction in the dihydroorotase protein quantity can be found. This clear connection identifies dihydroorotase unambiguously as novel target protein for herbicidal active ingredients, see Examples 3-7.

To allow effective inhibitors of plant dihydroorotase to be found, suitable test systems must be provided with which inhibitor-enzyme binding studies can be carried out. To this end, for example, the complete cDNA sequence of Solanum tuberosum dihydroorotase is cloned into an expression vector (pQE, Qiagen) and overexpressed in E. coli, see Example 8.

Alternatively, however, the expression cassette comprising a DNA sequence SEQ-ID No. 1 can be expressed, for example, in other bacteria, in yeasts, fungi, algae, plant cells, insect cells or mammalian cells.

The dihydroorotase protein expressed with the aid of the expression cassette according to the invention is particularly suitable for finding dihydroorotase-specific inhibitors.

To this end, the dihydroorotase can be employed, for example, in an enzyme test in which the dihydroorotase activity in the presence and absence of the active ingredient to be tested is determined. By comparing the two activity determinations, a qualitative and quantitative statement can be made on the inhibitory behavior of the active ingredient to be tested.

The enzymatic detection developed hitherto for measuring the dihydroorotase activity by the method of Mazus and Buchowicz (Acta Biochimica Polonica (1968), 15 (4), 317-325) is based on detecting the orotate formed in a dihydroorotate-dehydrogenase-coupled reaction mixture at 280 nm. This assay is not suitable for mass screening. The method was therefore designed in such a way that NADH formed can be detected at 340 nm. To do this, a high activity of the auxiliary enzyme, the dihydroorotate dehydrogenase, is required. A commercially available preparation from Zymobacterium oroticum (Sigma) proved to be too impure for the NADH formation to be monitored. In order to be able to carry out mass screening, the specific dihydroorotate dehydrogenase activity must be at least ten times higher than that in the commercial preparation. Such an activity was obtained by isolating a plant dihydroorotate dehydrogenase and expressing it in yeast (Saccharomyces cerevisiae). This is why a test system was developed which was based on coupling plant dihydroorotase and plant dihydroorotate dehydrogenase. To this end, for example the gene encoding an Arabidopsis thaliana dihydroorotate/

dehydrogenase was isolated (see Genbank Acc. No. x62909, Minet et al., Plant J. (1992), 2 (3), 417-422; Examples 9 - 11.

The test system according to the invention allows a large number of chemical compounds to be tested simply and rapidly for herbicidal properties. The method allows reproducibly to select in a directed fashion, from a multitude of substances, those with high potency in order to use these substances for subsequently carrying out other in-depth tests with which the skilled worker is familiar.

The invention furthermore relates to a method of identifying herbicidally active substances which inhibit the dihydroorotase activity in plants, consisting of the following steps

a) the generation of transgenic plants, plant tissues or plant cells which comprise an additional DNA sequence encoding an enzyme with dihydroorotase activity and which are capable of overexpressing an enzymatically active dihydroorotase;

b) applying a substance to transgenic plants, plant cells, plant tissues or plant parts and to untransformed plants, plant cells, plant tissues or plant parts;

c) determining the growth or the viability of the transgenic and the untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance; and

d) comparing the growth or the viability of the transgenic and the untransformed plants, plant cells, plant tissues or plant parts after application of the chemical substance;

where suppression of the growth or the viability of the untransformed plants, plant cells, plant tissues or plant parts without greatly suppressing the growth or the viability of the transgenic plants, plant cells, plant tissues or plant parts confirms that the substance of b) shows herbicidal activity and inhibits the dihydroorotase enzyme activity in plants.

The invention furthermore relates to a method of eliminating undesired vegetation, which comprises treating the plants to be eliminated with a compound which specifically binds to, and inhibits the function of, dihydroorotase encoded by a DNA sequence SEQ-ID No. 1 or a DNA sequence hybridizing with this DNA sequence.

The present invention furthermore relates to herbicidally active compounds which can be identified with the above-described test system.

Herbicidally active dihydroorotase inhibitors can be employed as defoliants, desiccants, haulm killers and, in particular, as weed killers. Weeds are to be understood as meaning, in the broadest sense, all plants which grow in locations where they are undesired. Whether the active ingredients found with the aid of the test system according to the invention act as total or selective herbicides depends, inter alia, on the quantity applied.

For example, herbicidally active dihydroorotase inhibitors can be used against the following weeds:

Dicotyledonous weeds of the genera:
Sinapis, Lepidium, Galium, Stellaria, Matricaria, Anthemis, Galinsoga, Chenopodium, Urtica, Senecio, Amaranthus, Portulaca, Xanthium, Convolvulus, Ipomoea, Polygonum, Sesbania, Ambrosia, Cirsium, Carduus, Sonchus, Solanum, Rorippa, Rotala, Lindernia, Lamium, Veronica, Abutilon, Emex, Datura, Viola, Galeopsis, Papaver, Centaurea, Trifolium, Ranunculus, Taraxacum.

Monocotyledonous weeds of the genera:
Echinochloa, Setaria, Panicum, Digitaria, Phleum, Poa, Festuca, Eleusine, Brachiaria, Lolium, Bromus, Avena, Cyperus, Sorghum, Agropyron, Cynodon, Monochoria, Fimbristyslis, Sagittaria, Eleocharis, Scirpus, Paspalum, Ischaemum, Sphenoclea, Dactyloctenium, Agrostis, Alopecurus, Apera.

The present invention also relates to expression cassettes whose sequences encode a Solanum tuberosum dihydroorotase or its functional equivalent. The nucleic acid sequence can be, for example, a DNA or a cDNA sequence.

In addition, the expression cassettes according to the invention comprise regulatory nucleic acid sequences which govern the expression of the coding sequence in the host cell. In accordance with a preferred embodiment, an expression cassette according to the invention comprises upstream, i.e. at the 5′-end of the coding sequence, a promoter and downstream, i.e. at the 3′-end, a polyadenylation signal and, if appropriate, other regulatory elements which are operably linked with the coding sequence, for the dihydroorotase gene, which is located in between. Operable linkage is to be understood as meaning the sequential arrangement of promoter, coding sequence, terminator and, if appropriate,

other regulatory elements in such a way that each of the regulatory elements can fulfill its intended function when the coding sequence is expressed.

An expression cassette according to the invention is generated by fusing a suitable promoter with a suitable dihydroorotase DNA sequence and a polyadenylation signal using customary recombination and cloning techniques as they are described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

The sequence homology between Solanum tuberosum dihydroorotase and Arabidopsis thaliana dihydroorotase is 78% identity at protein level. The homology was obtained using the program BLASTP (Altschul et al., Nucleic Acids Res. (1997) 25, 3389-3402), see Example 2.

The present invention also relates to functionally equivalent DNA sequences which encode a dihydroorotase gene and which, based on the total length of the gene, show 40 to 100% sequence homology with the DNA sequence SEQ-ID NO: 1.

Preferred subject matter of the invention are functionally equivalent DNA sequences which encode a dihydroorotase gene and which, based on the total length of the gene, show 60 to 100% sequence homology with the DNA sequence SEQ-ID NO: 1.

Particularly preferred subject matter of the invention are functionally equivalent DNA sequences which encode a dihydroorotase gene and which, based on the total length of the gene, show 80 to 100% sequence homology with the DNA sequence SEQ-ID NO: 1.

Functionally equivalent sequences which encode a dihydroorotase gene are, in accordance with the invention, those sequences which still have the desired functions, despite a differing nucleotide sequence. Functional equivalents thus encompass naturally occurring variants of the sequences described herein, and also artificial, for example chemically synthesized, artificial [sic] nucleotide sequences adapted to suit the codon usage of a plant.

A functional equivalent is also to be understood as meaning, in particular, natural or artificial mutations of an originally isolated, dihydroorotase-coding sequence which continues to show the desired function. Mutations encompass substitutions, additions, deletions, exchanges or insertions of one or more nucleotide residues. Thus, for example, the present invention also encompasses those nucleotide sequences which are obtained by modifying this nucleotide sequence. The aim of such a modification can be, for example, to further delimit the coding sequence contained therein, or else, for example, insert more restriction enzyme cleavage sites.

Functional equivalents are also those variants whose function is weaker or stronger in comparison with the original gene or gene fragment.

In addition, the expression cassette according to the invention can also be employed for the transformation of bacteria, cyanobacteria, yeasts, filamentous fungi and algae with the purpose of producing sufficient amounts of the enzyme dihydroorotase.

The present invention furthermore relates to a Solanum tuberosum protein which comprises the amino acid sequence SEQ-ID NO:2 or derivatives or parts of this protein with dihydroorotase activity. In comparison with the Arabidopsis thaliana dihydroorotase, the homology at amino acid level is 78% identity.

The present invention also relates to plant proteins with dihydroorotase activity with an amino acid sequence homology to the Solanum tuberosum dihydroorotase of 20 - 100% identity.

Preferred plant proteins with dihydroorotase activity are those with an amino acid sequence homology to the Solanum tuberosum dihydroorotase of 50 - 100% identity.

Particularly preferred plant proteins with dihydroorotase activity are those with an amino acid sequence homology to the Solanum tuberosum dihydroorotase of 80 - 100% identity.

It is another object of the present invention to overexpress the dihydroorotase gene in plants in order to generate plants which tolerate dihydroorotase inhibitors.

Overexpressing the dihydroorotase-encoding gene sequence SEQ-ID NO: 1 in a plant results in an increased resistance to dihydroorotase inhibitors. The present invention also relates to

the transgenic plants generated thus.

The expression efficacy of the transgenically expressed dihydroorotase gene can be determined, for example, in vitro by shoot meristem multiplication, or by a germination test. Also, an altered expression type and expression level of the dihydroorotase gene and their effect on the resistance to dihydroorotase inhibitors may be tested on test plants in greenhouse experiments.

The present invention furthermore relates to transgenic plants transformed with an expression cassette according to the invention comprising the DNA SEQ-ID No. 1, which plants have been made tolerant to dihydroorotase inhibitors by additional expression of the DNA sequence SEQ-ID No. 1, and to transgenic cells, tissues, parts and propagation material of such plants. Especially preferred are transgenic crop plants such as, for example, barley, wheat, rye, maize, soybeans, rice, cotton, sugar beet, canola, sunflowers, flax, hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce, and the various tree, nut and grapevine species, and also legumes.

The invention furthermore relates to plants, which, after expression of the DNA SEQ ID NO:1 in the plant, show an increased UMP content.

Increasing the uridine-5′-phosphate (UMP) content means, for the purposes of the present invention, the artificially acquired capability of an increased UMP biosynthesis performance by functionally overexpressing the dihydroorotase gene in the plant compared to the non-genetically-engineered plant for at least one plant generation.

Especially preferred sequences are those which ensure targeting into the apoplast, into plastids, into the vacuole, into the mitochondrium or into the endoplasmatic reticulum (ER) or which, due to a lack of suitable operative sequences, ensure that the product remains in the compartment of formation, the cytosol (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR (see Example 3).

A suitable promoter of the expression cassette according to the invention is, in principle, any promoter which is capable of governing the expression of foreign genes in plants. In particular, a plant promoter or a promoter derived from a plant

virus is preferably used. Especially preferred is the cauliflower mosaic virus CaMV 35S promotor (Franck et al., Cell 21(1980), 285-294). This promoter contains various recognition sequences for transcriptional effectors which in their totality lead to permanent and constitutive expression of the introduced gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette according to the invention may also comprise a chemically inducible promoter which allows expression of the exogenous dihydroorotase gene in the plant to be governed at a particular point in time. Such promoters, for example the PRP1 promotor (Ward et al., Plant.Mol. Biol. (1993) 22, 361-366), a salicylic-acid-inducible promoter (WO 95/1919443), a benzenesulfonamide-inducible promoter (EP 388186), a tetracyclin-inducible promoter (Gatz et al., Plant J. (1992) 2, 397-404), an abscisic-acid-inducible promoter (EP0335528) or an ethanol- or cyclohexanone-inducible promoter (WO 93/21334) are described in the literature and can be used, inter alia.

Furthermore, especially preferred promoters are those which ensure expression in tissues or parts of the plant in which the biosynthesis of purins or their precursors takes place. Promoters which ensure leaf-specific expression may be mentioned in particular. Promoters which may be mentioned are the potato cytosolic FBPase or the potato ST-LSI promoter (Stockhaus et al., EMBO J., (1989) 8, 2445-251 [sic]).

A foreign protein can be expressed stably in the seeds of transgenic tobacco plants to an extent of 0.67% of the total soluble seed protein with the aid of a seed-specific promoter (Fiedler and Conrad, Bio/Technology (1995) 10, 1090-1094). The expression cassette according to the invention can therefore comprise, for example, a seed-specific promoter (preferably the phaseolin promotor, the USP or LEB4 promotor), the LEB4 signal peptide, the gene to be expressed, and an ER retention signal.

The inserted nucleotide sequence encoding a dihydroorotase can be generated synthetically or obtained naturally or comprise a mixture of synthetic and natural DNA components. In general, synthetic nucleotide sequences are generated which have codons which are preferred by plants. These codons which are preferred by plants can be determined by codons with the highest protein frequency which are expressed in most of the plant species of interest. When preparing an expression cassette, it is possible to manipulate various DNA fragments so as to obtain a nucleotide sequence which expediently reads in the correct direction and which is equipped with a correct reading frame. To link the DNA

fragments to each other, adapters or linkers may be attached to the fragments.

Other suitable DNA sequences are artificial DNA sequences as long as they mediate, as described above by way of example, the desired property of increasing the UMP content in the plant by overexpressing the dihydroorotase gene in crop plants. Such artificial DNA sequences can be determined, for example, by backtranslating proteins which have been constructed by means of molecular modeling and which exhibit dihydroorotase activity, or by *in vitro* selection. Especially suitable are encoding DNA sequences which have been obtained by backtranslating a polypeptide sequence in accordance with the host-plant-specific codon usage. The specific codon usage can be determined readily by a skilled worker familiar with plant-genetic-engineering methods by means of computer evaluations of other, known genes of the plant to be transformed.

Further suitable equivalent nucleic acid sequences according to invention which may be mentioned are sequences which encode fused proteins, component of the fused protein being a plant dihydroorotase polypeptide or a functionally equivalent portion thereof. The second portion of the fused protein can be, for example, a further enzymatically active polypeptide or an antigenic polypeptide sequence with the aid of which detection for dihydroorotase expression is possible (for example myc-tag or his-tag). However, it is preferably a regulatory protein sequence such as, for example, a signal or transit peptide, which leads the dihydroorotase protein to the desired site of action.

Expediently, the promoter regions according to the invention and the terminator regions should be provided, in the direction of transcription, with a linker or polylinker comprising one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, in most cases 1 to 8, preferably 2 to 6, restriction sites. In general, the linker within the regulatory regions has a size less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter according to the invention can be native, or homologous, or else foreign, or heterologous, to the host plant. The expression cassette according to the invention comprises, in the 5′-3′-direction of transcription, the promoter according to the invention, any sequence and a region for transcriptional termination. Various termination regions may be exchanged for each other as desired.

Manipulations which provide suitable restriction cleavage sites or which eliminate the excess DNA or excess restriction cleavage

sites may also be employed. *In vitro* mutagenesis, prime repair, restriction or ligation may be used in cases where insertions, deletions or substitutions such as, for example, transitions and transversions, are suitable. Complementary ends of the fragments may be provided for ligation in the case of suitable manipulations such as, for example, restriction, chewing back or filling in overhangs for blunt ends.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which correspond essentially to *Agrobacterium tumefaciens* T-DNA polyadenylation signals, in particular those of gene 3 of the T-DNA (octopine synthase) of the Ti-plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 ff), or functional equivalents.

For transforming a host plant with a dihydroorotase-encoding DNA, an expression cassette according to the invention is incorporated, as insertion, into a recombinant vector whose vector DNA comprises additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described, inter alia, in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chapter 6/7, pp.71-119.

The transfer of foreign genes into the genome of a plant is termed transformation. It exploits the above-described methods for transforming and regenerating plants from plant tissues or plant cells for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic method using the gene gun, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and agrobacterium-mediated gene transfer. The abovementioned methods are described in, for example, B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus Annu. Rev. Plant Physiol.Plant Molec.Biol. 42 (1991) 205-225). The construct to be expressed is preferably cloned into a vector which is suitable for the transformation of *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711).

Agrobacteria transformed with an expression cassette according to the invention can equally be used in a known manner for transforming plants, in particular crop plants such as cereals, maize, soybeans, rice, cotton, sugar beet, canola, sunflowers, flax, hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce and the various tree, nut and grapevine species, and

legumes, for example by bathing wounded leaves or leaf sections in an agrobacterial suspension and subsequently growing them in suitable media.

The biosynthesis site of pyrimidines is, generally, the leaf tissue, so that leaf-specific expression of the dihydroorotase gene is useful. However, it is obvious that the pyrimidine biosynthesis need not be limited to the leaf tissue, but may also take place in all other remaining parts of the plant in a tissue-specific fashion, for example in fatty seeds.

Moreover, constitutive expression of the exogenous dihydroorotase gene is advantageous. On the other hand, inducible expression may also be desirable.

Using the above-cited recombination and cloning techniques, the expression cassettes according to the invention can be cloned into suitable vectors which allow them to be multiplied, for example in E. coli. Suitable cloning vectors are, inter alia, pBR332, pUC series, M13mp series and pACYC184. Especially suitable are binary vectors, which are capable of replication both in E. coli and in agrobacteria.

The present invention furthermore relates to the use of an expression cassette according to the invention for the transformation of plants, plant cells, plant tissues or parts of plants. The preferred aim of the invention is to increase the dihydroorotase content in the plant.

Depending on the choice of the promoter, expression may take place specifically in the leaves, in the seeds or other parts of the plant. Such transgenic plants, their propagation material and their plant cells, tissue or parts are a further subject of the present invention.

The invention is illustrated by the examples which follow, but not limited thereto:

Examples

Genetic engineering methods on which the use examples are based:

General cloning methods

Cloning methods such as, for example, restriction cleavage, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes,

linking DNA fragments, transformation of Escherichia coli cells, growing bacteria, and the sequence analysis of recombinant DNA, were carried out as described by Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6).

Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using an ABI laser fluorescence DNA sequencer following the method of Sanger (Sanger et al. (1977), Proc. Natl. Acad. Sci. USA74, 5463-5467). Fragments resulting from a polymerase chain reaction were sequenced and checked in order to avoid polymerase errors in constructs to be expressed.

Example 1

Isolation of a cDNA encoding a functional plant dihydroorotase

A clone encoding dihydroorotase was obtained from potatoes by functional complementation of an E.coli mutant. The mutant used was the mutant CGSC5152 (CS101-2U5) of the E. coli Genetic Stock Center, which carries a mutation in the pyrC gene locus encoding a dihydroorotase. Complementation was effected by electrotransformation of competent cells of strain CGSC5152 with a cDNA library in the vector plasmid pBS SK-. The underlying lambda ZAPII library (Stratagene) was cloned in an undirected fashion with EcoRI/NotI linkers following standard procedures. The RNA template for the cDNA was isolated from sink leaves (small 1-cm-leaflets harvested from 10-week-old potato plants, grown in the greenhouse).

The transformed E. coli cells were plated on M9 minimal medium (Sambrook et al., 1989) complemented with methionine (20 mg/l), ampicillin (100 mg/l) and IPTG (2.5 mM). In total, 4 micrograms of the library were transformed in 8 batches, giving rise to 36 clones which, following examination by means of restriction cleavage, proved to be identical.

Example 2

Sequence analysis of the cDNA clones encoding a protein with dihydroorotase activity

The resulting 36 cDNA clones encode a polypeptide with homology to dihydroorotases from other organisms. The homology was obtained using the program BLASTP (Altschul et al., Nucleic Acids Res. (1997) 25, 3389-3402). Accordingly, the protein has 78% identity with Arabidopsis thaliana dihydroorotase, 58% identity with Synechocystis dihydroorotase, 55% identity with E. coli and Pseudomonas putida dihydroorotase. The longest clone was termed pyrCSt5. The plasmid was given the name pBSSK-pyrCSt5. The cDNA (see SEQ-ID No. 1) has an open reading frame of 1046 base pairs with a stop sodon in position 1047-1049. The amino acid sequence starts with the third base in the reading frame and can be translated into a polypeptide 348 amino acids in length (see SEQ-ID No. 2). This corresponds to the length of prokaryotic dihydroorotase-coding sequences.

Owing to the reading frame of the present cDNA sequence, it cannot be deduced with certainty whether it might possibly be a form localized in the plastids or a cytosolic form.

Example 3

Generation of plant expression cassettes

A 35S CaMV promoter was inserted into plasmid pBin19 (Bevan et al., Nucl. Acids Res. 12 (1980), 8711) in the form of an EcoRI-KpnI fragment (corresponding to nucleotides 6909-7437 of the cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285). The polyadenylation signal of gene 3 of the T-DNA from Ti-plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984), 835), nucleotides 11749-11939 was isolated as a PvuII-HindIII fragment and, after addition of SphI linkers, cloned into the PvuII cleavage site between the SphI-HindIII cleavage site of the vector. This gave rise to plasmid pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990), 221-230). Cloning of a construct of pyrCSt5 in antisense orientation in pBinAR was done by an Asp718 cleavage site (internal cleavage site of 964 bp) and a BamHI cleavage site (from the polylinker).

Example 4

Generation of transgenic potato plants

Potato plants (cv. Solara) were transformed with the aid of Agrobacterium tumefaciens using the corresponding construct pBinAR-anti-pyrCSt5. The plasmid was transformed into Agrobacterium tumefaciens C58C1:pGV2260 (Deblaere et al., Nucl. Acids. Res. 13 (1984), 4777-4788). To transform potatoes by the method of Rocha-Sosa et al. (EMBO J., 8 (1988), 23-29), a 1:50 dilution of an overnight culture of a positively transformed agrobacterial colony in Murashige-Skoog medium (Physiol. Plant., 15 (1962), 473) was used. Leaf disks of sterile plants (in each case approx. 1 cm^2) were incubated for 5-10 minutes in a 1:50 agrobacterial solution in a petri dish. This was followed by incubation in the dark for 2 days at 20°C on MS medium. Cultivation was subsequently continued in a 16 hour light/8 hour dark photoperiod. For shoot induction, explants were transferred weekly to MS medium supplemented with 500 mg/l claforan (cefotaxime-sodium), 50 mg/l kanamycin and plant hormones (Rocha-Sosa et al., EMBO J., 8, 23-29, 1989) and 1.6 g/l glucose. Growing shoots were transferred to MS medium supplemented with 2% sucrose, 250 mg/l claforan and 0.8% Bacto-agar.

Regenerated shoots are obtained on 2MS medium supplemented with kanamycin and claforan, transferred into the soil after they have struck roots and, after culture for two weeks in a controlled-environment cabinet in a 16-hour-light/8-hour-dark photoperiod at an atmospheric humidity of 50%, examined for expression of the foreign gene, altered metabolite contents and phenotypic growth characteristics. Altered nucleotide contents may be determined, for example, by the method of Stitt et al. (FEBS Letters, 145 (1982), 217-222).

Example 5

Analysis of total RNA from plant tissues

Total RNA from plant tissues was isolated as described by Logemann et al., Anal. Biochem. 163 (1987), 21. For the analysis, in each case 20 micrograms of RNA were separated in a formaldehyde-containing 1.5% strength agarose gel and transferred to Duralon UV membranes (Stratagene).

To detect specific transcripts, digoxygenine-labeled probes were prepared by means of PCR following the manufacturer's instructions and used for hybridization (DIG EasyHyb,

Boehringer). Then, the membranes were washed for 3 x 20 minutes in wash buffer (2x SSC, 0.1% SDS) at 60°C. Detection was carried out by luminescence and exposure to Hyperfilm ECL (Amersham) using the Boehringer DIG detection system with CDP-Star as substrate.

Resulting individual transgenic plants of lines ROSa-34, -31, -10, -19, -9 and -3 are shown in Figure 3 as test plants at RNA level. A band is recognizable at 1.6 kb in accordance with the expected dihydroorotase transcript size and, in the case of plants ROSa-3, -9, -31, -34, the 1.1 kb antisense transcript. A marked reduction in RNA quantity can be found, in particular, in the case of plant ROSa-9.

Example 6

Detection of the potato dihydroorotase protein in tuber and leaf tissues.

To generate a polyclonal serum against the dihydroorotase polypeptide, a peptide sequence from the potato dihydroorotase amino acid sequence was chosen. The peptide LGTDSAPHDRRRKEC was synthesized by a commercial company (Eurogentec, Seraing, Belgium) and coupled to KLH (keyhole limpet protein) via the C-terminal cysteine. The conjugate was employed, again, by the commercial company (Eurogentec) for immunizing rabbits and antisera against the peptide were obtained. In Western blot experiments, the antiserum specifically recognizes the potato polypeptide. To this end, protein was subjected to an SDS polyacrylamide gel electrophoresis under denaturing conditions, transferred to nitrocellulose membranes and detected by means of immunodetection following the manufacturer's instructions (ECL-System, Amersham). Transgenic plants of the ROSa lines were characterized with the aid of the antiserum. Lines -3, -9 and -40 show different degrees of protein reduction in the leaf, see Figure 2. Plant -40 does not form tubers. Plants -3 and -9 also show a correspondingly greatly reduced dihydroorotase protein quantity in tubers.

Example 7

Phenotypic analysis of transgenic plants.

Plants of lines ROSa, which carry a dihydroorotase antisense construct were characterized in greater detail. The plants show differing degrees of growth retardation. Plant line ROSa-40 is affected to such an extent that no tubers are formed. Plants of

this line are not viable in the greenhouse and must be maintained in vitro. A correlation can be found between growth retardation and reduction in dihydroorotase protein quantity. This clear connection identifies potato dihydroorotase unambiguously as novel target protein for herbicidal active ingredients.

Example 8

Generation of overexpression vectors in E. coli

The following oligonucleotide sequences were derived from the sequence determined, and provided with a BamHI restriction cleavage site and with two base overhangs.

1. 5'-primer aaggatccGCAAAAATGGAGCTCTCA

2. 3'-primer aaggatccTCAGAGAGGAGCCGGCAAC

The PCR reaction mixtures contained 8 ng/μl pBSSK-pyrCSt5 DNA, 0.5 μM of the corresponding oligonucleotides, 200 μM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 1.5 mM $MgCl_2$ and 0.02 U/μl Taq polymerase (Perkin Elmer). The amplification conditions were set as follows:

Denaturation temperature:	92°C, 1 min
Annealing temperature:	52°C, 1 min
Elongation temperature:	72°C, 2.5 min
Number of cycles:	30

The PCR fragments were cloned into the overexpression vector pQE9 via BamHI and employed for protein production by means of IPTG induction following standard methods (see Handbuch: The QiaExpressionist, Qiagen, Hilden).

Example 9

Test system for measuring the dihydroorotase activity

The enzymatic detection developed to date for measuring the dihydroorotase activity by the method of Mazus and Buchowicz, (Acta Biochimica Polonica (1968), 15(4), 317-325) is based on detecting the orotate formed at 280 nm in a dihydroorotate-dehydrogenase-coupled reaction mixture. Prerequisite for doing so is a high activity of the auxiliary enzyme, viz. dihydroorotate dehydrogenase. A commercially available preparation from Zymobacterium oroticum (Sigma) proved

to be too contaminated.

In order to be able to carry out a mass screening, the specific dihydroorotate dehydrogenase activity must be at least ten times higher than is the case in the commercial preparation. Such an activity was obtained by preparing a dihydroorotate dehydrogenase activity from Neurospora crassa (R.W. Miller, Methods in Enzymology LI, 1978, 63 - 69) after cloning a plant dihydroorotate dehydrogenase and its expression in yeast (Saccharomyces cerevisiae). A further improvement of the test system was achieved by carrying out the measurement at 340 nm.

First, an Arabidopsis thaliana dihydroorotate dehydrogenase was isolated (see Genbank Acc. No. X62909, Minet et al., Plant J. (1992), 2 (3), 417-422).

The following oligonucleotide sequences were derived from the database entry of the dihydroorotate dehydrogenase sequence:

1. 5'-primer aaggatccatggccggaagggctg

2. 3'-primer aaggatccttagtggtggtggtggtggtgtttgtgggatggggc

The PCR reaction mixtures contained 10 ng of plasmid DNA from an Arabidopsis thaliana cDNA in vector pFL61 (ATCC 77600), 0.5 microM [sic] of the corresponding oligonucleotides, 200 μM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 1.5 mM $MgCl_2$ and 0.02 U/μl Taq polymerase (Perkin Elmer). The amplification conditions were set as follows:

Denaturation temperature:	92°C, 0.5 min
Annealing temperature:	60°C, 0.5 min
Elongation temperature:	72°C, 1.5 min
Number of cycles:	35

The resulting PCR fragment was first cloned into the yeast expression vector pYES2 (Invitrogen) via the BamHI cleavage sites. The construct generated was named pYES2-pyrDAt.

Example 10

Cloning of a plant dihydroorotate dehydrogenase from tobacco

Furthermore, the PCR fragment described in Example 9 was applied for a heterologous screening in a tobacco phage cDNA library. The cDNA employed for generating the tobacco phage cDNA library was obtained from RNA from tobacco cell suspension cultures. The cDNA

library was generated following the manufacturer's instructions (Stratagene). 3.0 x 10^5 lambda phages of the Nicotiana tabacum cDNA library were plated on agar plates with E. coli XLI-Blue as bacterial strain.

The phage DNA was transferred to nylon filters (Duralon UV, Stratagene) by means of standard methods (Sambrook et al. (1989); Cold Spring Harbor Laboratory Press: ISBN 0=87969-309-6) and fixed on the filters. The hybridization probe used was the above-described PCR fragment, which was DIG-labeled with the aid of the labeling and detection system (Boehringer, Mannheim) following the manufacturer's instructions. Hybridization of the membrane was carried out for 16 hours at 42°C in DIG EasyHyb (Boehringer). The filters were subsequently washed for 3 x 20 minutes in 2 x SSC, 0.1 % SDS at 60°C. Positively hybridizing phages were on Hyperfilm ECL (Amersham) by luminescence with the Boehringer DIG detection system using CDP-Star as substrate, and purified and isolated by standard techniques.

Ten identical clones resulted, of which clone pyrDT10 was sequenced completely (SEQ-ID No. 3). An EcoRI digest of the clone shows an EcoRI fragment 1962 base pairs in size with an open reading frame of 458 amino acids, a start codon in position 305-307 and a stop codon in position 1679-1681. The deduced amino acid sequence (SEQ-ID No. 4) of the tobacco dihydroorotate dehyrogenase exhibits 72% identity with the Arabidopsis amino acid sequence, 51% identity with the rat amino acid sequence, 43% identity with the yeast amino acid sequence, 37% identity with the E. coli amino acid sequence. The identity was obtained using the program BLASTP (Altschul et al., Nucleic Acids Res. (1997) 25, 3389-3402).

The following oligonucleotide sequences were derived from the sequence determined, and provided with a KpnI restriction cleavage site and two base overhangs.

1. 5'-primer ggggtaccatgagacaaagggttggatt

2. 3'-primer ggggtaccttagtggtggtggtggtggagaggagccggcaacca

The PCR reaction mixtures contained 5 ng/µl pBSSK-pyrDT10 DNA, 0.5 µM of the corresponding oligonucleotides, 200 µM nucleotides (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C), 1.5 mM $MgCl_2$ and 0.02 U/µl Taq polymerase (Perkin Elmer). The amplification conditions were set as follows:

Denaturation temperature: 92°C, 1 min

Annealing temperature:	52°C, 1 min
Elongation temperature:	72°C, 2.5 min
Number of cycles:	30

The PCR fragment of the tobacco dihydroorotate dehydrogenase was cloned into the yeast expression vector pYES2 (Invitrogen) via KpnI cleavage sites. This construct (pYES-pyrDT10) and the Arabidopsis dihydroorotate dehydrogenase construct pYES2-pyrDAt were inserted into the ural yeast mutant for complementation (Minet et al., Gene (1992), 121(2), 393-6). Resulting yeast clones were grown in liquid culture overnight in complete medium supplemented with 1% galactose.

Example 11

Enzyme isolation of plant dihydroorotase and dihydroorotate dehydrogenase, and measurement of the dihydroorotase activity

The dihydroorotase E.coli expression cultures, and the yeast expression culture containing the tobacco (or Arabidopsis) dihydroorotate dehydrogenase, were in each case disrupted separately by means of pressure disruption methods using the French Press under maximum pressure in a 20 ml pressurized chamber, or with the aid of a glass ball mill (IMA Desintegrator). Per 1 g of cell pellet, 10 ml of buffer (0.1M KH_2PO_4; pH 7.5; 0.4M sucrose, 0.1 mM DTT) are used. By adding a 2.5-fold amount of glass beads (d=0.5mm), the pellet is disrupted in the glass ball mill for 20 minutes at 4°C and 2500 rpm. The batch is centrifuged for 20 minutes at 4°C and 100,000g. The enzyme activity was determined in a photometric assay by measurement in a photometer (Uvikon 933, Kontron) at 340 nm. The choice of the overexpression vectors also allowed the dihydroorotase and the dihydroorotate dehydrogenase to be purified via the histidin anchor by standard methods in one step under native conditions if the disruption buffer was free from DTT (cf. also Handbuch: The QiaExpressionist, Qiagen, Hilden). The eluates were subjected to dialysis to change the buffer to 20 mM potassium phosphate buffer pH 6.1; 5 mM $MgCl_2$; 1 mM DTT; 10 mM cysteine; 10 μM $ZnCl_2$, 20 μM NAD. In each case 10-100μl of the resulting enzyme fraction was made up with buffer to 700 μl and measured against a reference cell containing 700 μl reaction buffer and 100 μl of a protein homogenate of untransformed E. coli culture. The reaction was started using 7 mM carbamyl aspartate. Identical quantities of total protein were employed for measuring the untransformed or transformed E. coli extracts.

As an alternative to plant dihydroorotate dehydrogenase activities expressed in yeasts, it is possible to employ a dihydroorotate dehydrogenase activity prepared from Neurospora crassa, see R.W. Miller, Dihydroorotate dehydrogenase, (in: Methods in Enzymology 51 (1978), 63 - 69).

Alternatively, the dihydroorotase may also be measured in a less sensitive colorimetric assay by the method of Prescott and Jones (Anal. Biochem. (1969) 32, 408-419) without being coupled to dihydroorotate dehydrogenase. To this end, the dihydroorotase activity was measured in 50 mM Tris-HCl, 1 mM dihydroorotate (pH 8.5) after incubation at 37°C by detecting the carbamoyl aspartate formed. Prerequisite to this is the protein preparation with high protein activity which has been described in this example.

The potato dihydroorotase activity measured in the assay systems described can be reduced with known dihydroorotase inhibitors such as 6-L-thiodihydroorotate or 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate (Christopherson et al., Biochemical Society Transactions 23: 888-893, 1995).